Fragmentation of Phosphopeptides in an Ion Trap Mass Spectrometer

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A systematic study of the fragmentation pattern of phosphopeptides in an electrospray (ESI) ion trap mass spectrometer is presented. We show that phosphotyrosine- and phosphothreonine-containing peptides show complicated fragmentation patterns. These phosphopeptides were observed to lose the phosphate moiety in the form of H_3PO_4 and/or HPO_3, but were also detected with no loss of the phosphate group. The tendency to lose the phosphate moiety depends strongly on the charge state. Thus, the highest observed charge state tends to retain the phosphate moiety with extensive fragmentation along the peptide backbone. We also show that phosphoserine-containing peptides have relatively simple fragmentation patterns of losing H_3PO_4. This loss is independent of the charge state. We suggest strategies for the accurate identification of phosphorylation sites using the ion trap mass spectrometer. (J Am Soc Mass Spectrom 1998, 9, 1175–1188) © 1998 American Society for Mass Spectrometry

Tandem mass spectrometry is becoming the method of choice for the identification of phosphorylation sites due to its reliability, sensitivity, and speed [1–11]. Multiply charged phosphopeptide ions generated by electrospray ionization (ESI) are significantly easier to fragment than corresponding singly charged ions, and the phosphate group is less likely to be lost than from singly charged phosphopeptides generated by matrix-assisted laser desorption/ionization (MALDI) under collision-induced-dissociation (CID) conditions [11, 12]. Using the triple-stage-quadrupole (TSQ) mass spectrometer, and more recently the quadrupole ion trap mass spectrometer, we and others showed that fragmentation of phosphopeptides in tandem mass spectrometry is a general route for determination of phosphorylation sites [9, 13]. In contrast, only limited success has been shown for the decomposition of singly charged phosphopeptide ions generated by MALDI with MALDI/time-of-flight (TOF) instruments operated in postsource decay (PSD) mode and in MALDI/ion trap instruments [8, 10, 11].

The ion trap mass spectrometer shows great promise for the identification of phosphorylation site with high sensitivity [13]. We recently developed a strategy that uses phosphatase treatment with MALDI-TOF to identify phosphopeptides in a protein digest, followed by ion trap mass spectrometry to identify the precise phosphorylation sites [13]. This strategy allows the identification of phosphorylation sites from phosphopeptides that cannot even be detected in the normal (single-stage) mass spectrum. Ion trap mass spectrometers are also unique in that the time scales for measuring and exciting ions in tandem mass spectrometry (MS/MS) mode are much longer than for nontrapping mass spectrometers. This allows the observation of decomposition channels that require less energy but longer time and, because of this, it was observed that phosphopeptides lose predominantly 98 Da (H_3PO_4) when subjected to MS/MS in ion trap mass spectrometers [14–16]. This mass loss often serves as a signature for phosphopeptides.

In this paper, we report a study of fragmentation of peptides containing phosphoserine, phosphothreonine, and phosphotyrosine in an ion trap mass spectrometer. We show that, in contrast to phosphoserine-containing peptides, phosphothreonine- and phosphotyrosine-containing peptides undergo complicated fragmentation on losing the phosphate moiety, and the loss of the phosphate moiety is charge state dependent.

Experimental

Materials and Sample Preparation

The expressed catalytic domain of myosin I heavy chain kinase (MIHCK) of *Acanthamoeba castellanii* was purified as described previously [17]. The phosphopeptide with the sequence of RA**p**SVVGTTYWMAPEVVK (**p**S denoting phosphoserine) were generated with in-solution digestion of MIHCK with sequencing grade modified trypsin (Boehringer Mannheim, Indianapolis, IN) in 50 mM NH_4HCO_3 for 2 h at 37°C. Phosphopeptides with the sequences of RASVVGT**p**TYWMAPEVVK, RASVVG**p**TTYWMAPEVVK, **p**SPQPLGGSHR, SPQPLGG**p**SHR, TNQG**p**YFFFHLPD-NH_2, ERTMPRIPTLKNLED-

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LVTE**p**YHGNFSAW-NH_2, GDAGSN**p**TINASY-NH_2, pyroERRIDTLNSDG**p**YTPEPARITSPD-NH_2, and FAGV EAGGARAPQPSSVV**p**YR were synthesized by the Tufts University peptide sequencing core facility (Boston, MA). The phosphopeptide IIESYEGNS**p**YTFIDPTQLPYNEK-WEFPR was generated by in-gel digestion of the rat colony stimulation factor-1 receptor (CSF-1R). The phosphopeptide LFTGHPE**p**TLEK was obtained from the 1998 mass spectrometry survey of the Association of Biomolecular Resource Facilities (ABRF).

Electrospray/LC/Ion Trap and Nanospray Ion Trap

The electrospray liquid chromatography (LC) ion trap (LCQ) system has been described previous [13]. A second LCQ coupled with a nanospray ion source (The Protein Analysis Company, Odense M, Denmark) was also used to perform MS^n (n = 2,3) experiments. One to 2 μL of the synthetic phosphopeptide samples dissolved in 50% methanol and 10% acetic acid were used directly for nanospray. Samples requiring desalting were done utilizing the technique described by Mann [18]. Ions were isolated with a mass isolation window of up to 4 Da for +2 charged ions, but this window was reduced to 3 Da for ions of higher charge states. Spectra were taken in profile mode in order to allow inspection of peak shapes and accurate determination of isotopic peak patterns when using nanospray. The automatic gain control (AGC) was not used in experiments that required better control of the ion population to maximize sensitivity. The collision energy parameter was chosen to be the minimum needed to promote extensive fragmentation while maximizing ion signal (usually around 30% of the highest available collision energy as defined in the LCQ control software for multiply charged ions).

Results and Discussion

Phosphoserine loses H_3PO_4 through β-elimination to produce dehydroalanine. Phosphoserine-containing peptides were tested, and it was found that they all lose predominantly a moiety with a mass of 98 Da in ion trap mass spectrometry, in agreement with previous findings [13–16]. For example, the peptide, RA**p**SVVGTTYWMAPE-VVK (monoisotopic mass 1972.98 Da), which is phosphorylated at the serine residue [14], shows one transition m/z 987.5 to 938.7 (loss of 49 Da) (Figure 1a). A high-resolution scan (Zoom Scan) confirms that the ion of m/z 938.7 is doubly charged. Furthermore, most of the b-type ions [19] are not observed; instead ions with masses of $b_n - 98$ Da are found for the fragments that contain the phosphoserine (see b_4^Δ, b_5^Δ, b_{10}^Δ to b_{12}^Δ, and b_{14}^Δ to b_{16}^Δ), showing their loss of H_3PO_4. Of course, the y-type ions are observed because they do not contain the phosphoserine (see y_5 to y_7, y_9, and y_{12} to y_{14} ions). Only one fragment ion, b_{12}, resulting from cleavages at the preferential residue of proline contains the phosphate group.

Because we did not observe fragment ions generated from cleavages on both sides of the phosphoserine, positive identification of the product of phosphoserine after losing the moiety of H_3PO_4 was not possible. To investigate further the phosphorylation of this peptide, we carried out a MS^3 experiment by isolating and fragmenting the product ions of m/z 938.7 (Figure 1b). Now that the facile loss of the phosphate group has occurred, we see extensive fragmentation along the peptide backbone. A series of y ions are observed, of which y_{15} at m/z 1648.8 and y_{14} at m/z 1579.5 (giving a mass difference of 69.3 Da) unequivocally identify the third residue as a dehydroalanine (69.1 Da).

The above experiment provides another route for identifying phosphorylation sites. Locating phosphoserine as a dehydroalanine becomes important when the phosphate group is so unstable that it is lost in preference to peptide-backbone cleavage, as was observed in MALDI/ion trap experiments [11]. In an electrospray ion trap mass spectrometer, we found that loss of H_3PO_4 for multiply charged phosphopeptides does not usually pose a serious problem for the identification of phosphorylation sites in MS^2 experiments. Although fragmentation along the peptide backbone occurs, it can be much less facile than the loss of H_3PO_4. Identification becomes difficult when two or more serine/threonine/tyrosine residues are so closely spaced that cleavage between them does not occur.

Loss of H_3PO_4 from phosphoserine does not depend on the charge state. Because electrospray generates ions with a distribution of charge states, we decided to examine how the charge states influence the fragmentation of phosphopeptides to choose the most appropriate one to fragment. We found that ions of all available charge states of phosphoserine-containing peptides lose H_3PO_4 and give some peptide backbone fragmentation. This is illustrated for **p**SPQPLGGSHR (monoisotopic mass 1114.53 Da). The singly and doubly charged ions lose H_3PO_4 and fragment along the peptide backbone (Figure 2a, b). Similarly, the triply charged ion (the highest charge state) loses primarily H_3PO_4, but less peptide backbone fragmentation occurs than with the singly and doubly charged ions (Figure 2c). The three charge states of a second peptide with the same sequence except with phosphorylation at the second serine (SPQ-PLGG**p**SHR) show dominant losses of H_3PO_4. Other phosphoserine-containing peptides (data not shown) confirmed that ions of different charge states also fragment with similar 98-Da losses, suggesting that the loss of H_3PO_4 from phosphoserine-containing peptides is not charge state dependent.

For some phosphoserine-containing peptides, the 98-Da loss is not as dominant as that seen in Figures 1a and 2. Nevertheless, these peptides did lose the H_3PO_4. Two phosphopeptides of sequence SPQPLGGSHR, where phosphorylation is at different serines, both exhibit 98-Da loss. The peptide with phosphorylation at the second serine, however, exhibits greater fragmenta-

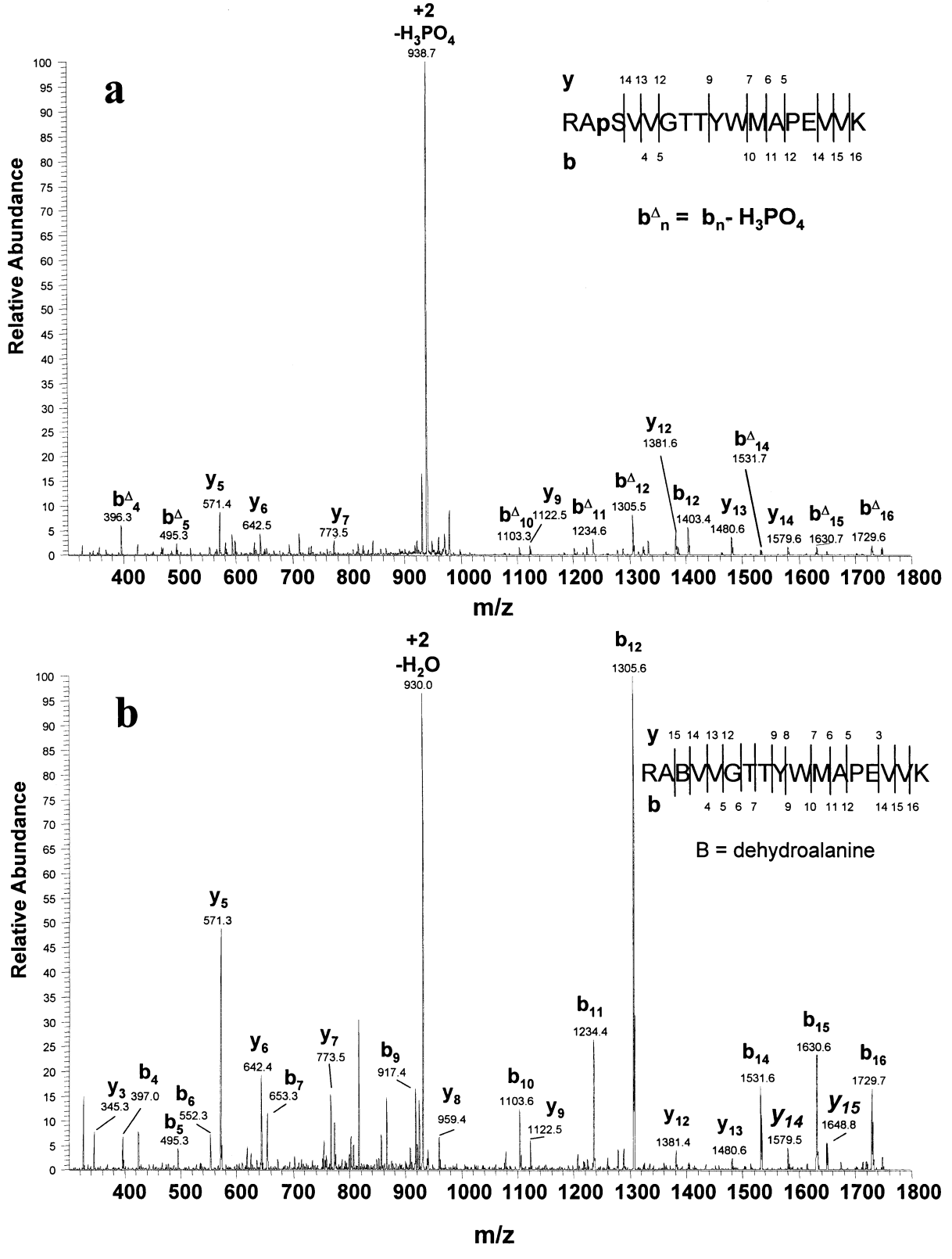

Figure 1. Phosphoserine loses predominantly H_3PO_4 through β elimination to produce dehydroalanine in tandem ion trap mass spectrometry. (**a**) MS^2 spectrum of a doubly charged phosphopeptide ion (m/z 987.5). A loss of 98 Da (H_3PO_4) is observed. The b_n^{Δ} label denotes loss of 98 Da (i.e., b_n-H_3PO_4). (**b**) MS^3 spectrum of the ion arising from loss of 98 Da [m/z 938.7 of the doubly charged ion in (a)]. The y_{14} and y_{15} fragments have a mass difference of 69 Da that corresponds to the mass of dehydroalanine, identifying the product of phosphoserine after losing 98 Da as dehydroalanine. The "B" label denotes the dehydroalanine residue.

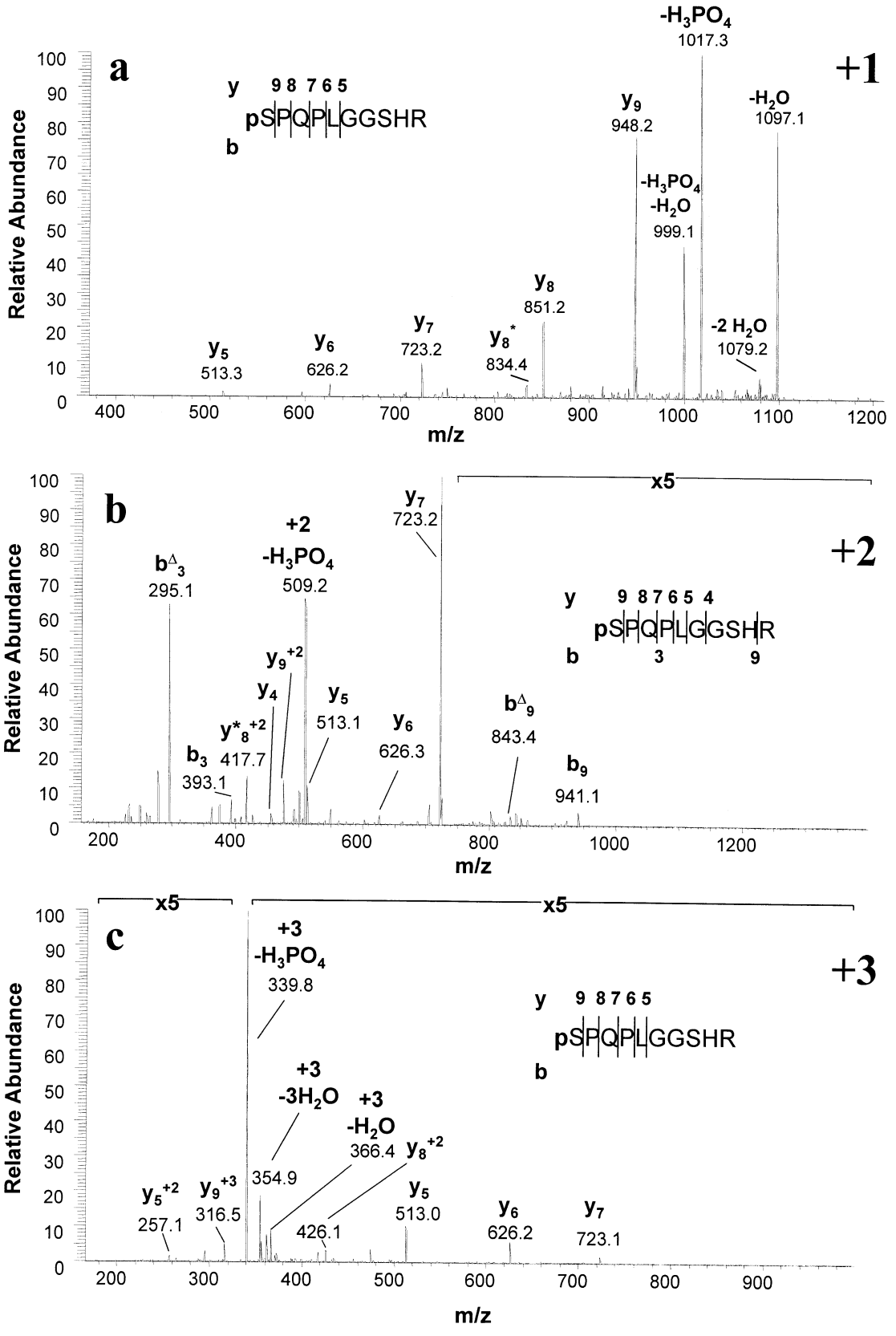

Figure 2. Loss of H_3PO_4 is not charge state dependent for phosphoserine-containing peptides. (**a**) MS^2 spectrum of the singly charged ion (m/z 1115.5). (**b**) MS^2 spectrum of the doubly charged ion (m/z 558.3). (**c**) MS^2 spectrum of the triply charged ion (m/z 372.5). Losses of H_3PO_4 (98 Da) are observed in fragmentation of all charge states. Fragments with asterisk (e.g., y_n^*) represent loss of 17 Da (presumably NH_3) from the corresponding b_n or y_n fragment.

tion of the peptide backbone for all charge states. Providing that another parameter exists that can influence fragmentation of phosphoserine-containing peptides, the distance between the phosphoserine residue and the residue that carries the charge (for example, arginine at the C terminus) may be a factor in this fragmentation, but this has not yet been evaluated.

Phosphotyrosine-containing peptides can lose H_3PO_4. Phosphotyrosine-containing peptides cannot undergo the same β-elimination reaction as phosphoserine- or phosphothreonine-containing peptides. Phosphotyrosine-containing peptides, however, can lose a neutral with the mass of 98 Da [16]. Our experiments, also in an ion trap, with a wider range of phosphotyrosine-containing peptides, showed similar results. For example, the loss was observed in the product–ion spectrum of the triply charged pyro-ERRIDTLNSDG**p**YTPEPARITSPD-NH_2 (monoisotopic mass 2664.26 Da, see Figure 3). Zoom scans conclusively established that the loss was indeed 98 Da. Strong losses of multiples of 18 Da (presumably of H_2O) also occur. This loss was usually not observed for the phosphoserine-containing peptides, and it contrasts with the fragmentation of phosphotyrosine-containing peptides induced by high-energy collisions in a magnetic-sector instrument and with low-energy CID in a triple-stage quadrupole instrument, where phosphotyrosine-containing peptides often lose 80 Da [1, 3]. More backbone fragmentation occurs for phosphotyrosine-containing peptides, often making the identification of the tyrosine phosphorylation site easier than that of serine. In contrast to fragments of phosphoserine-containing peptides, the fragments containing the phosphotyrosine residue do not usually lose H_3PO_4 (see y_{13}, y^*_{13}, b^{*+2}_{13}, b^{+2}_{13}, b^{+2}_{15}, b^{+2}_{19}, b^{+2}_{21}, b^{*+2}_{21} in Figure 3).

Phosphotyrosine-containing peptides may lose HPO_3. We found one example of a phosphotyrosine-containing peptide, FAGVEAGGARAPQPSSVV**p**YR (monoisotopic mass 2098.03 Da, Figure 4), that undergoes a loss of 80 Da (HPO_3), in agreement with what has been observed in both magnetic-sector and triple-stage quadrupole instruments [1, 3]. This peptide also loses 98 Da, and the backbone fragmentation is extensive. The fragments containing the phosphotyrosine residue do not subsequently lose the phosphate group (note all the y ions).

Phosphotyrosine-containing peptides may not lose H_3PO_4 or HPO_3. We were surprised to observe that phosphotyrosine-containing peptides can fragment without loss of the phosphate group as H_3PO_4 or HPO_3. Instead, they fragment only along the peptide backbone. Figure 5a shows the product–ion spectrum of the +3 charged peptide ions of IIESYEGNS**p**YTFIDPTQLPYNEKWEFPR (monoisotopic mass 3515.63 Da), and Figure 5b shows the +1 charged peptide ions of GDAGSNpYINASY-NH_2 (monoisotopic mass 1309.52 Da). In neither case did losses of 98 or 80 Da occur.

The origin of the 80- and 98-Da loss for phosphotyrosine-containing peptides. The fragmentation pattern of the peptide as shown in Figure 4 provides an illustrative example for determining the origin of the 80- and 98-Da losses by MS^3. The ion corresponding to the 80-Da loss (m/z 1010.0 as shown in Figure 4) was subjected to an additional stage of fragmentation (MS^3, see Figure 6a) to give nearly exclusive fragmentation along the peptide backbone. The b_{19} (m/z 1844.5) and b_{18} (m/z 1681.6) establish that the product formed by loss of 80 Da is tyrosine.

We observed the same 98-Da loss in a MALDI/ion trap mass spectrometer from phosphotyrosine-containing peptides [11]. We hypothesized that this loss arises from consecutive losses of HPO_3 (80 Da) and H_2O (18 Da), but this hypothesis was not tested [11]. Thus, we carried out a MS^3 experiment for the ion corresponding to the 98-Da loss (m/z 1001.0 in Figure 4) and its second generation product–ion spectrum is shown in Figure 6b. Surprisingly, two ions labeled $b^{\$}_{19}$ (m/z 1844.8), b_{18} (m/z 1681.7) were observed, identifying the product structure at the phosphotyrosine site as tyrosine. Another molecule of H_2O must be lost in the peptide to account for this mass difference of 18 Da. The mass of the $b^{\$}_{19}$ ion also locates the position of the H_2O loss at the arginine residue and the C terminus. The complimentary y^{Δ}_2 fragment (m/z 320.1, corresponding to the arginine and tyrosine minus H_2O) further confirmed this identification. The H_2O loss is not from the two serine residues even though peptides containing serine tend to lose H_2O, but instead from the arginine or more likely the C terminus. We could not distinguish the loss of 98 Da as a one-step loss of H_3PO_4 (analogous to β-elimination of phosphoserine-containing peptides) or a two-step loss of the HPO_3 group, followed by loss of the H_2O, as commonly occurring in CID of peptide ions. The above observation, however, is evidence against the two-step loss mechanism as dominant for the 98-Da loss, because loss of H_2O from the C terminus is not often observed under normal circumstances. A more likely mechanism for the net 98-Da loss is that H_3PO_4 is lost in one step through a rearrangement reaction. The long timescale of the ion trap mass spectrometer allows this type of rearrangement reaction to occur.

Loss of the phosphate from phosphotyrosine is charge state dependent. We were puzzled by the observation that phosphotyrosine-containing peptides sometimes do not lose the phosphate group at all. A study of phosphotyrosine-containing peptides with different charge states revealed that ions of the highest charge state do not produce a loss of either 98 or 80 Da. The product–ion spectra of a phosphopeptide of ERTMPRIPTLKNLEDLVTE**p**YHGNFSAW-NH_2 (monoisotopic mass 3295.60 Da) with the +2, +3 and +4 charge states are shown in Figure 7. The +2 and +3 charge states showed 98-Da

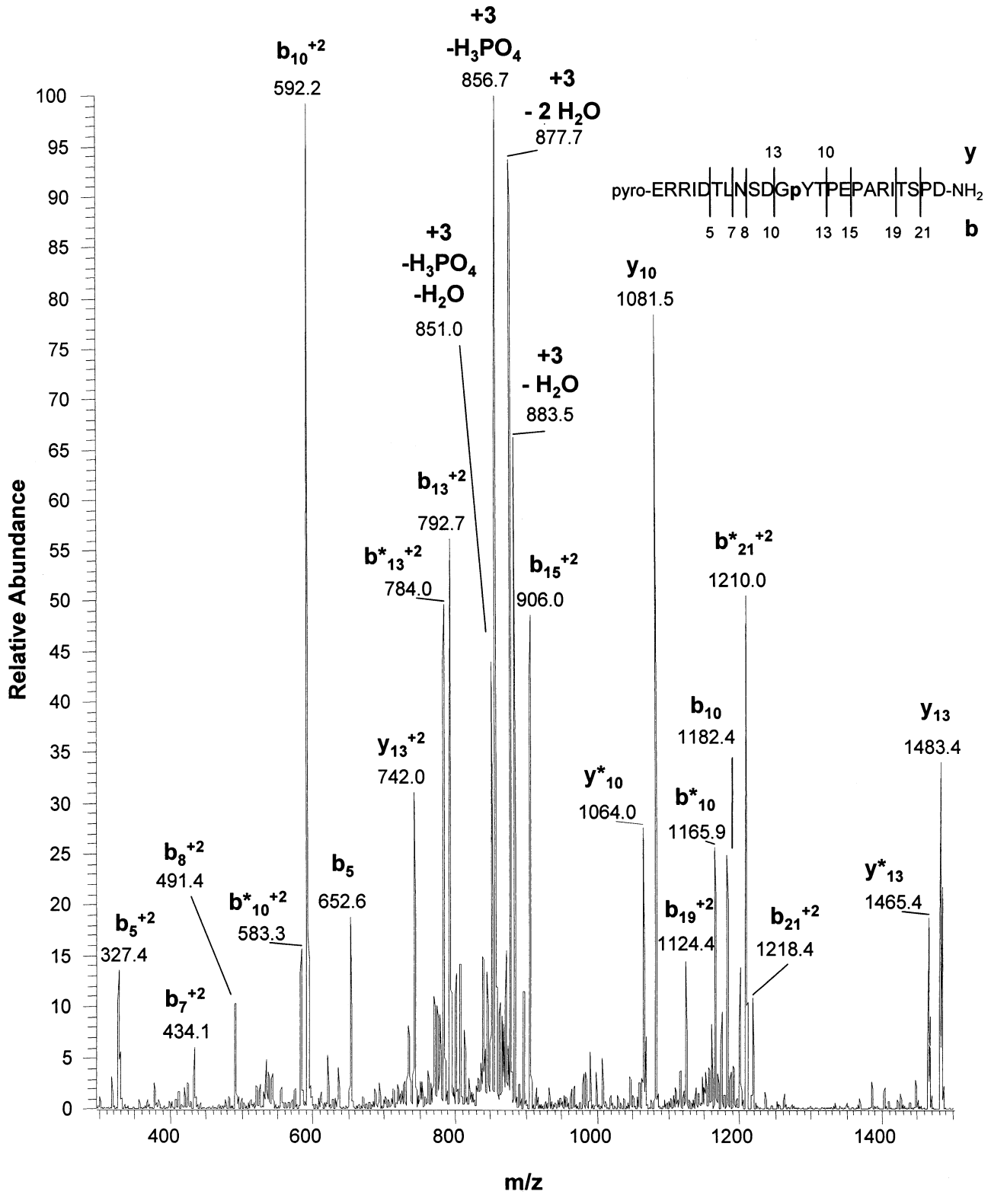

Figure 3. Phosphotyrosine-containing peptides may formally lose H_3PO_4 in tandem ion trap mass spectrometry. The MS/MS spectrum of a triply charged phosphotyrosine-containing peptide ion (m/z 889.1) of pyro-ERRIDTLNSDG**p**YTPEPARITSPD-NH_2. The C terminus is an amide. A loss of 98 Da is observed (see the peak corresponding to the ion at m/z 856.7).

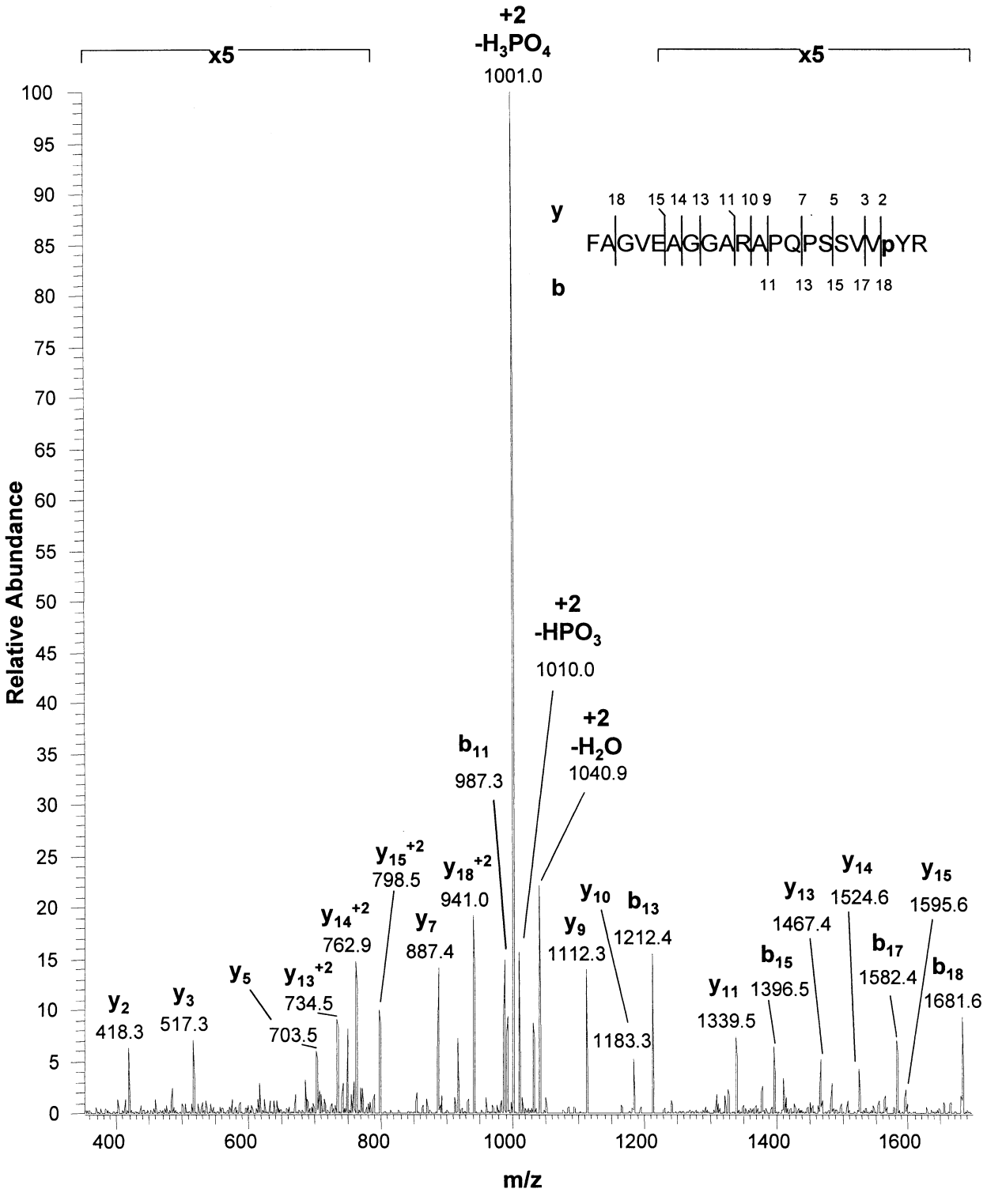

Figure 4. Phosphotyrosine-containing peptides may formally lose HPO_3. MS^2 spectrum of a doubly charged phosphotyrosine-containing peptide ion (m/z 1050.0). Losses of both H_3PO_4 and HPO_3 are observed. Note that fragment ions containing phosphotyrosine do not lose an additional H_3PO_4 moiety.

losses as expected (Figure 7a, b), whereas the +4 charge state (highest observed charge state) did not undergo this loss (Figure 7c). Rather, fragmentation occurs along the peptide backbone and resulted in a fragmentation pattern characteristic of unphosphorylated peptides. Similar results were also found for a range of phosphotyrosine-containing peptides (Table 1). The highest charge states of these peptides were either experimen-

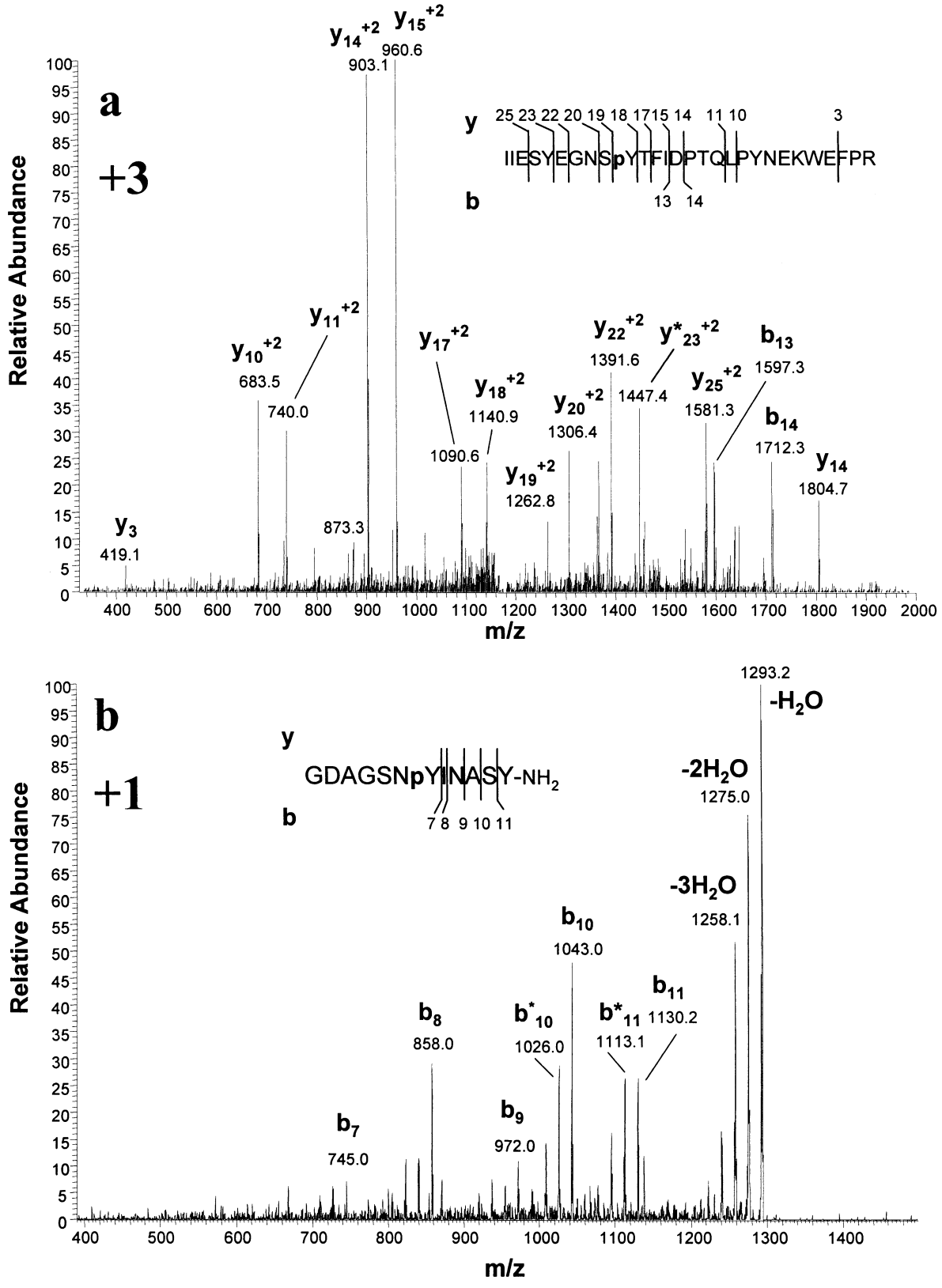

Figure 5. Phosphotyrosine-containing peptides may not lose the phosphate group. (**a**) LC/MS/MS spectrum of a triply charged phosphotyrosine-containing peptide ion (m/z 1172.9) of sequence IIESYEGNS**p**YTFIDPTQLPYNEKWEFPR. No observable loss of either H_3PO_4 (98 Da) or HPO_3 (80 Da) is apparent. (**b**) MS^2 spectrum of a singly charged phosphotyrosine-containing peptide ion (m/z 1310.5) of GDAGSN**p**YINASY-NH_2 by nanospray. The C terminus is an amide. Loss of water and some peptide backbone fragmentation are observed but no loss of H_3PO_4 (98 Da) or HPO_3 (80 Da) is observed.

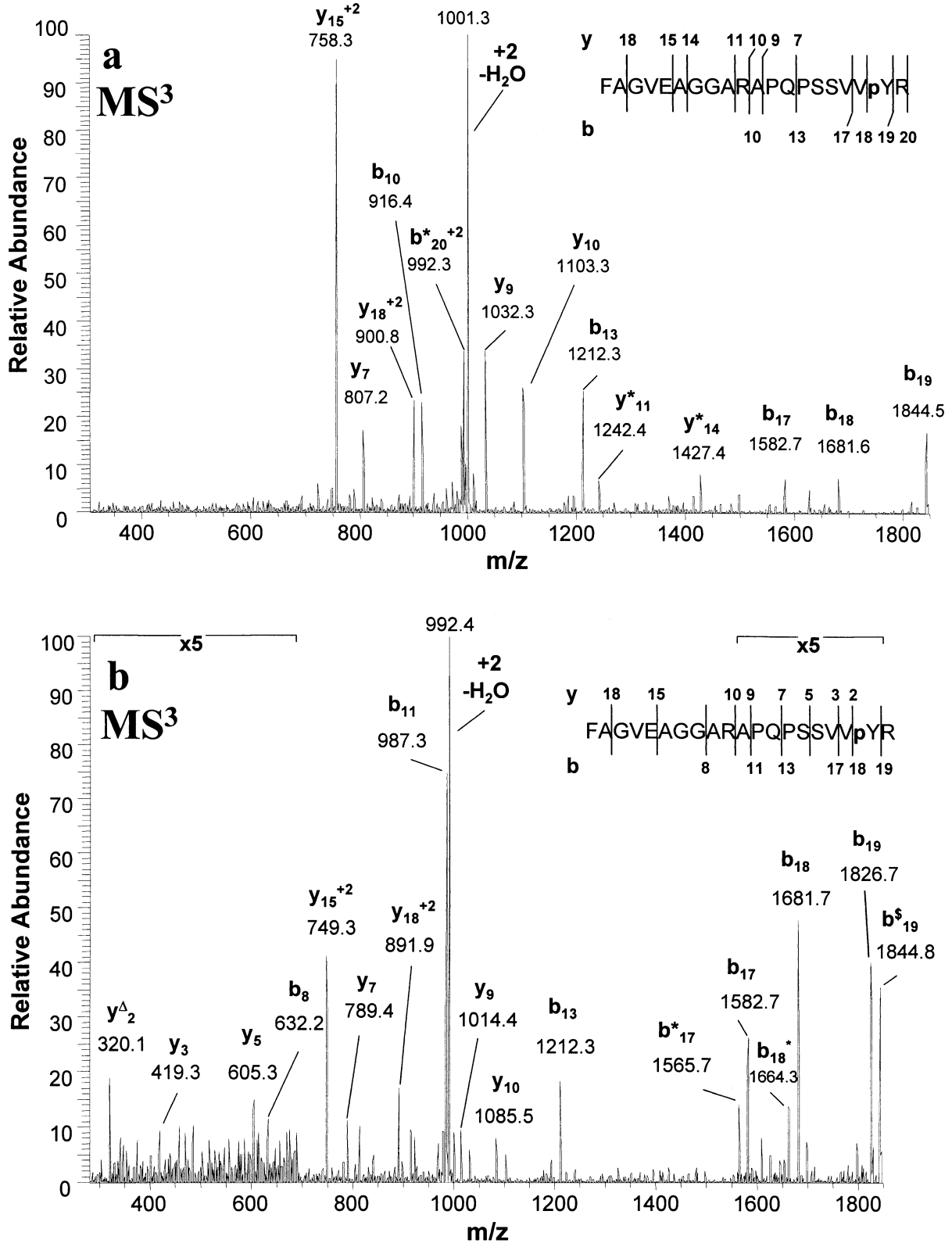

Figure 6. The origin of the 80- and 98-Da loss for phosphotyrosine containing peptides. (**a**) MS^3 spectrum of the ion (m/z 1010.0 in Figure 4) corresponding to the loss of HPO_3 from the doubly charged precursor ion (m/z 1050.0). The observed b_{19} and b_{18} ions identify the phosphotyrosine residue after losing 80 Da as tyrosine. (**b**) MS^3 spectrum of the ion (m/z 1001.0) corresponding to the loss of H_3PO_4 from the doubly charged precursor ion (m/z 1050.0 in Figure 4). The observed b_{19} and b_{18} identify the phosphotyrosine residue after losing 98 Da as tyrosine. The H_2O loss that accounts for the additional 18-Da loss must occur at the *R* residue or the C terminus. The y_2^{Δ} ion confirms this conclusion.

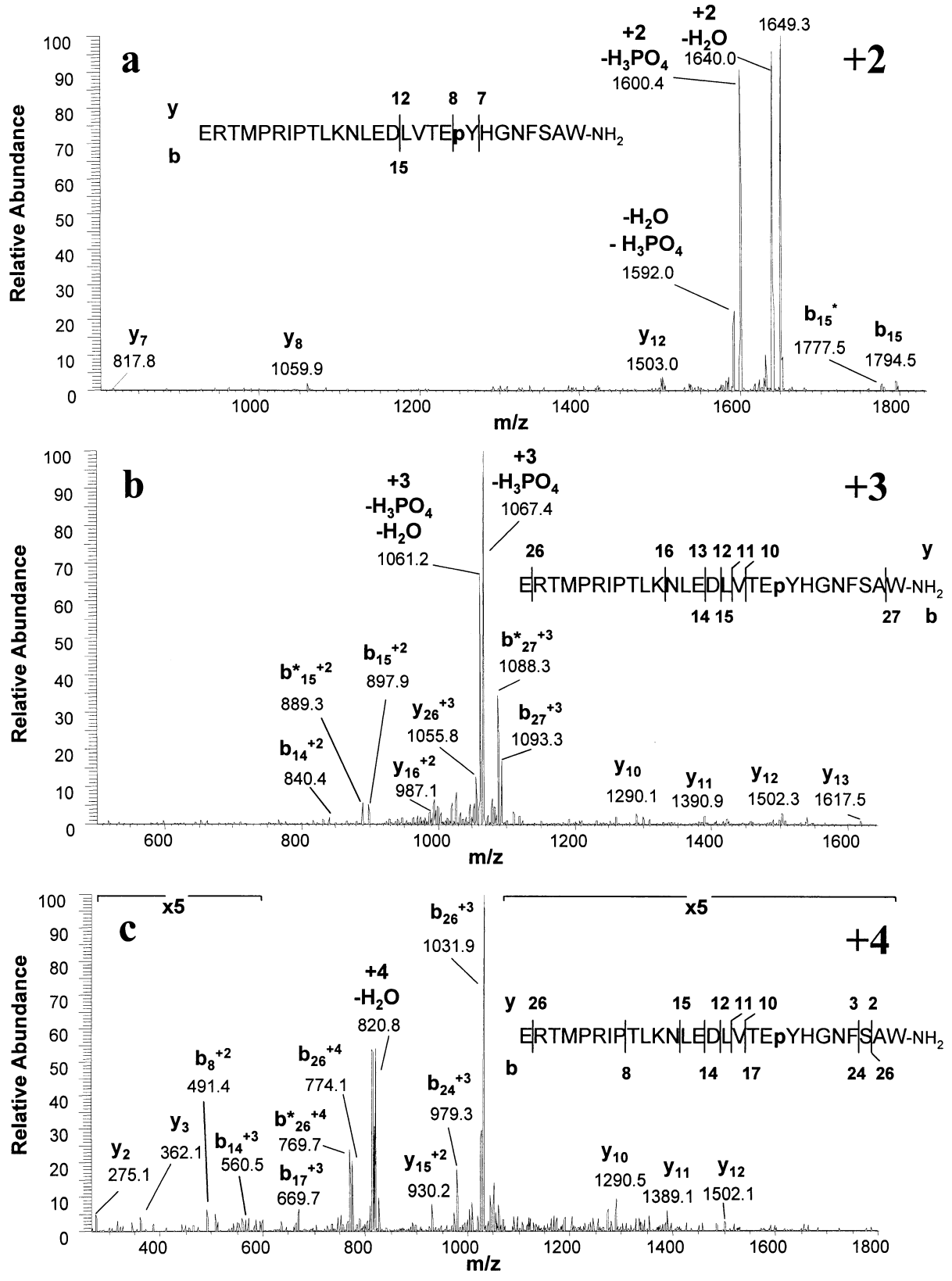

Figure 7. Loss of the phosphate moiety depends strongly on the charge state of phosphotyrosine-containing peptides. (**a**) MS^2 spectrum of the doubly charged ion (m/z 1648.8). A loss of H_3PO_4 is observed. (**b**) MS^2 spectrum of the triply charged ion (m/z 1099.5). A loss of H_3PO_4 is observed. (**c**) MS^2 spectra of the quadruply charged ion (m/z 824.9). A loss of H_3PO_4 or HPO_3 is not observed. Unlike phosphoserine-containing peptides, loss of the phosphate is charge state dependent. The C terminus is an amide.

Table 1. Summary of fragmentation of phosphotyrosine- and phosphothreonine-containing peptides

Sequence	Maximum charge	Phosphate loss[a]
IIESYEGNS**p**YTFIDPTQLPYNEKWEFPR	+3	None
ERTMPRIPTLKNLEDLVTE**p**YHGNFSAW-NH_2	+4	None
pyro-ERRIDTLNSDG**p**YTPEPARITSPD-NH_2	+4	None
QRRIDTLNSDG**p**YTPEPARITSPD	+4	None
TNQG**p**YFFFHLPD	+2	None
RASVVG**p**TTYWMAPEVVK	+3	None
RASVVGT**p**TYWMAPEVVK	+3	None
LFTGHPE**p**TLEK	+3	None

[a]None indicates no loss of H_3PO_4 from the maximum charge state but loss of H_3PO_4 from all lower-charge states.

tally observed or estimated from the number of chargeable amino acid residues (arginine, lysine, histidine, and N terminus). There might exist one charge difference between the observed highest charge state and the calculated one.

These results suggest that a competing fragmentation pathway exists, that it is charge-state dependent, and it somehow prevents elimination of the phosphate group. This observation is surprising, as the phosphate P–O bond is much weaker than the peptide bond. We hypothesize that the charged amino-acid residues interact with the phosphate group to stabilize it (perhaps in the form of a salt bridge [20]), resulting in a different chemical structure from the neutral peptide and the peptide ions of lower charge states. The long timescale in the ion trap may facilitate this process. As for phosphoserine-containing peptides, the phosphate is so unstable that the interaction with the charged group is insufficient to stabilize it.

Phosphothreonine loses H_3PO_4 through β-elimination and HPO_3 through dephosphorylation. Others have noted that fragmentation loss of H_3PO_4 from phosphothreonine-containing peptides is similar to that of phosphoserine-containing peptides [15]. Prompted by our observation of the diverse and complicated fragmentation patterns for phosphotyrosine-containing peptides, we performed a detailed investigation of phosphothreonine-containing peptides. Similar to phosphoserine-containing peptides, the +2 ion of RASVVGT**p**TYWMAPEVVK (monoisotopic mass 1972.98 Da) fragments with an appreciable loss of 98 Da (H_3PO_4), in agreement with previous findings (note the m/z 939 ion in Figure 8a). To our surprise, a careful inspection of the spectrum revealed an ion of m/z 947.5, which may form by loss of 80 Da (HPO_3). Some fragments containing the phosphothreonine residue lose the H_3PO_4 group (see the labeled b_n and b_n^{Δ} ions), placing the stability of the phosphothreonine residue between those of phosphoserine and phosphotyrosine.

To verify that the product formed by loss of 98 Da is the dehydroaminobutyric acid (a β-elimination), the ions formed by loss of H_3PO_4 were isolated and subjected to MS^3 (Figure 8b). The observed b_8 and b_7 ions of m/z 754.3 and 671.3 confirmed the product as the dehydroaminobutyric acid. This conclusion was further confirmed by the complimentary y ion series (see y_{10} and y_9 ions).

To examine further the product from the unexpected 80-Da loss, we isolated the ions from loss of HPO_3 and subjected them to MS^3 (Figure 8c). The mass difference (100.9 Da) between b_8 and b_7 ions identifies the residue as threonine, a product of dephosphorylation. Apparently, two competing channels operate, leading to the loss of H_3PO_4 and HPO_3. The same peptide when phosphorylated at the serine residue does not show any loss of 80 Da in MS^2 (see Figure 1a).

Loss of the phosphate from phosphothreonine is charge state dependent. Similar to phosphotyrosine-containing peptides, loss of the phosphate group for phosphothreonine containing peptides also depends on the charge state. The ions of the highest charge do not lose the phosphate group (see Table 1). Figure 9 shows the product–ion spectra of the peptide LFTGHPE**p**TLEK (monoisotopic mass 1350.66 Da) for the +2 (Figure 9a) and +3 (Figure 9b) states. Loss of 98 Da is observed for +2 but not for +3 (the highest observed charge state). This is similar to the fragmentation loss of 98 Da from phosphotyrosine-containing peptides.

Implications for the identification of phosphorylation sites. The evidence presented in this investigation suggests that the loss of 98 Da can always be used as a signature for phosphopeptides in an ion trap, however, the opposite is not always true. The lack of 98-Da loss from the highest charge state of phosphotyrosine or phosphothreonine-containing peptides cannot be used as evidence to assign them as unphosphorylated peptides. In addition, phosphorylation on serine, tyrosine, and threonine cannot generally be distinguished from each other based solely on an observed loss of H_3PO_4 (98 Da) from all charge states in tandem ion trap mass spectrometry. Is evidence available elsewhere in the spectrum? It may be possible to distinguish phosphoserine from the other two phosphoamino acids by the observed 98-Da loss if the highest observed charge state is fragmented. Loss of 98 Da from the highest charge state suggests the presence of a phosphoserine-containing peptide. Peptides containing these three phosphoamino acids may also be distinguished by their fragment ions that contain the phosphoamino acid. Phosphoserine-

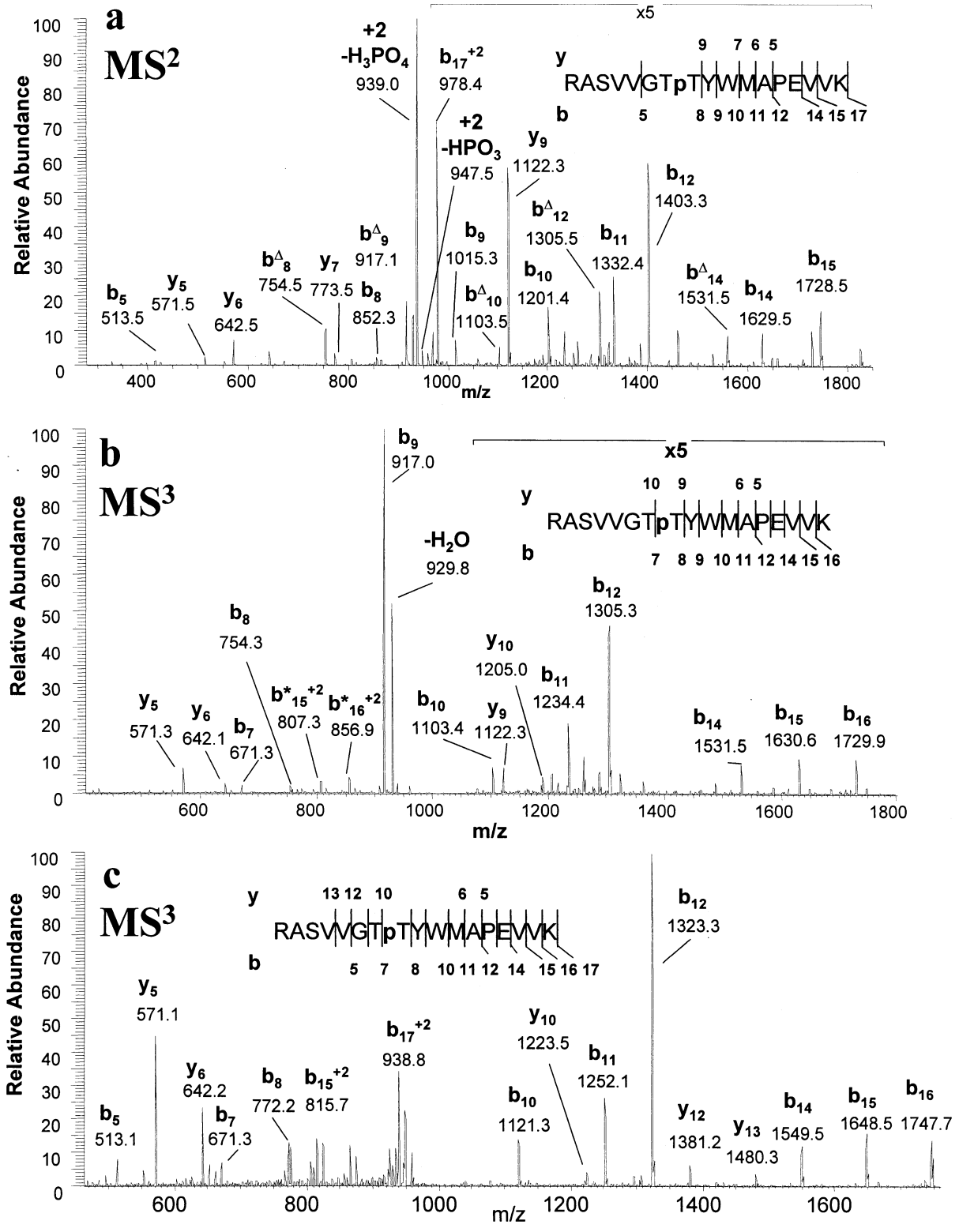

Figure 8. Phosphothreonine-containing peptides may lose H_3PO_4 through β elimination and HPO_3 through dephosphorylation. (**a**) MS^2 spectrum of the doubly charged phosphopeptide ion (m/z 987.6). Losses of both H_3PO_4 and HPO_3 are observed. Fragment ions containing phosphothreonine partially lose H_3PO_4. (**b**) MS^2 spectrum of the ion (m/z 939.0) corresponding to the loss of H_3PO_4 from the doubly charged precursor ion [m/z 987.6 of (a)]. The observed b_8 and b_7 identify the product of phosphothreonine after losing 98 Da as dehydroaminobutyric acid. The complimentary y_{10} and y_9 ions confirm this conclusion. (**c**) MS^3 spectrum of the ion [m/z 947.5 in (a)] corresponding to the loss of HPO_3 from the doubly charged precursor ion (m/z 987.6). The observed b_8 and b_7 identify the product of phosphothreonine after losing 80 Da as threonine.

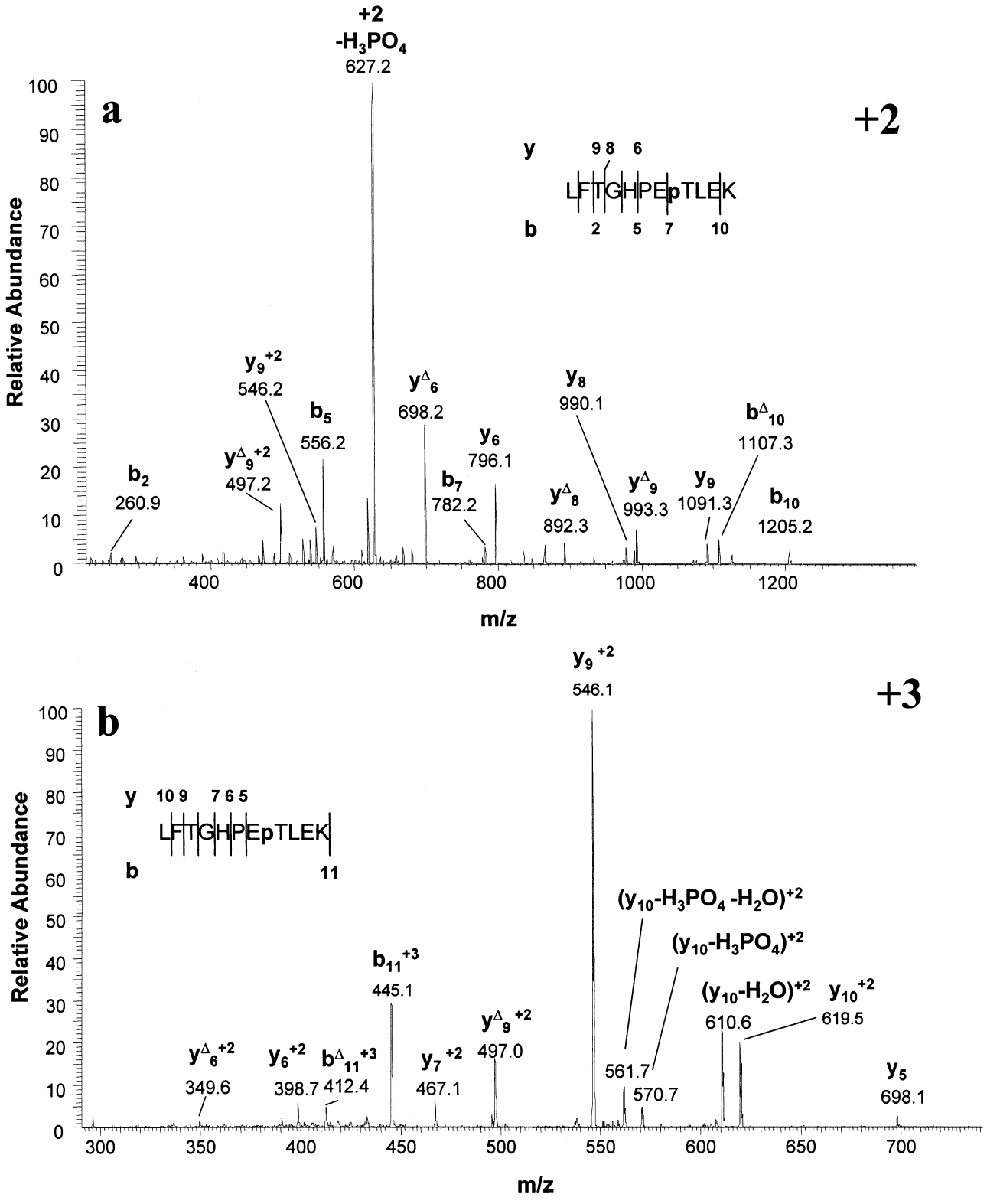

Figure 9. Loss of the phosphate moiety also depends on the charge state of phosphothreonine-containing peptides. (**a**) MS^2 spectrum of the doubly charged phosphothreonine-containing peptide ion (m/z 676.3). A dominant loss of H_3PO_4 is observed along with some peptide backbone fragmentation. The y_n^{Δ} label denotes loss of H_3PO_4 (i.e., y_n-H_3PO_4). (**b**) MS^2 spectra of the triply charged phosphothreonine-containing peptide ion (m/z 451.2). The +3 charge state is the highest charge state and shows no loss of 98 or 80 Da. Loss of 98 Da appears only for a few fragment ions. Like phosphotyrosine-containing peptides, loss of H_3PO_4 is charge state dependent.

containing fragments tend to lose H_3PO_4 completely, but phosphotyrosine-containing fragments do not lose H_3PO_4. Some phosphothreonine-containing fragments partially lose H_3PO_4, showing intermediate behavior. In any case, only through direct sequencing can the phosphorylation site be unambiguously determined.

What strategy is best to sequence phosphopeptides for the identification of phosphorylation sites? The results suggest that for phosphothreonine and phosphotyrosine-containing peptides, ions of the highest charge state might be a better choice for determining the phosphorylation site, as the highest charge states often produce more intensive fragmentation along the peptide backbone (see Figure 7a, b, and c). For phosphoserine-containing peptides, the charge state with the most intense peak should be chosen.

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